Montpellier, july, 27th, 2023

Dear recommender,

Please find here the answers of the authors to the recommenders' and the reviewers' comments. You will also find on BioRxiv (https://doi.org/10.1101/2023.05.09.539061) the revised version of our preprint (Influence of endosymbionts on the reproductive fitness of the tick *Ornithodoros moubata*)

We would like to thank you and the reviewers for taking time to give us essential and constructive comments to improve the quality of our preprint. Detailed responses to both the recommender's and reviewers' comments are attached above. We hope that the changes we have made following reviewer's queries and suggestions have improved the article and now ensured its appropriateness for recommendation in PCI Infections.

Recommender: Angélique Gobet

General comments:

Overall, the manuscript is well written but some parts may need some rearrangements to help the reading of the manuscript.

We fully agree with the recommender comment. The discussion has been rearranged to make it easier to access core information. The material and methods section has also been reorganised to make it clearer and more coherent with the order used in the rest of the preprint.

A general comment that has also been noted by Dr Raggi is that, when the authors used the term "microbiota", it is not clear whether they refer only to the targeted endosymbionts or to the whole microbial community associated with the ticks. In the text, it becomes clear only in the discussion L345-347, so the authors may be more specific earlier in the text.

We agree with the recommender and reviewer comment. The terms microbiota and endosymbionts have indeed been ambiguously used in the manuscript. The ambiguity comes from the fact that when performing antibiotic treatments, we targeted all bacterial members of the microbiota which were all probably sensitive to the antibiotics. However, without *a priori* sequencing methods, it was not possible to monitor all members of the microbiota. Based on previous studies, FLE and Rickettsia were the two predominant members of the microbiota of our strain of *Ornithodoros moubata* and were also the only primary symbionts identified for this tick species. They are now well known to have a crucial role in the tick development and survival and that is the reason why our study focus on these two symbionts even though it is probable that environmental microbiota was also impacted through our treatments. We clarified it and replaced "microbiota" by "FLE and Rickettsia" when appropriate in the new version of the manuscript.

The experimental set-up is very thorough but as suggested by Dr Aivelo, some rearrangements of the text may help the reading. For instance, in figure 1, the authors may assign a number to the different steps of the experiment and then refer to each step in the text.

As rightly suggested by Dr Aivelo, the Material and methods part has been reorganized. We also clarified the legend of figure 1 as suggested.

As Dr Aivelo suggested, some parts of the discussion may be trimmed and rearranged in other parts of the manuscript to make the reading easier.

We agree with the reviewer comment and as recommended, the discussion was rearranged to highlight core information and make it easier to read. Unnecessary information was removed and several parts were shortened.

Specific comments:

Please find below some specific comments complementary to those from the 2 reviewers:

L19-21: This sentence may be rephrased. With the later sentence introducing the prevalence of the endosymbionts, it is not clear whether the authors followed the whole microbiota or only the two endosymbionts.

As recommended, this was clarified. (Line 19)

L87: Are the "main bacterial species" Francisella-like and Rickettsia endosymbionts? Please specify.

Yes, the recommender is completely right, this refers to FLE and Rickettsia. This was specified in the text. (Line 93)

L92: Materials and methods should be written in the past tense.

This query has been addressed in the revised version of the manuscript.

L102: I was wondering if it would be of interest to specify if these stages were only female or of both sexes.

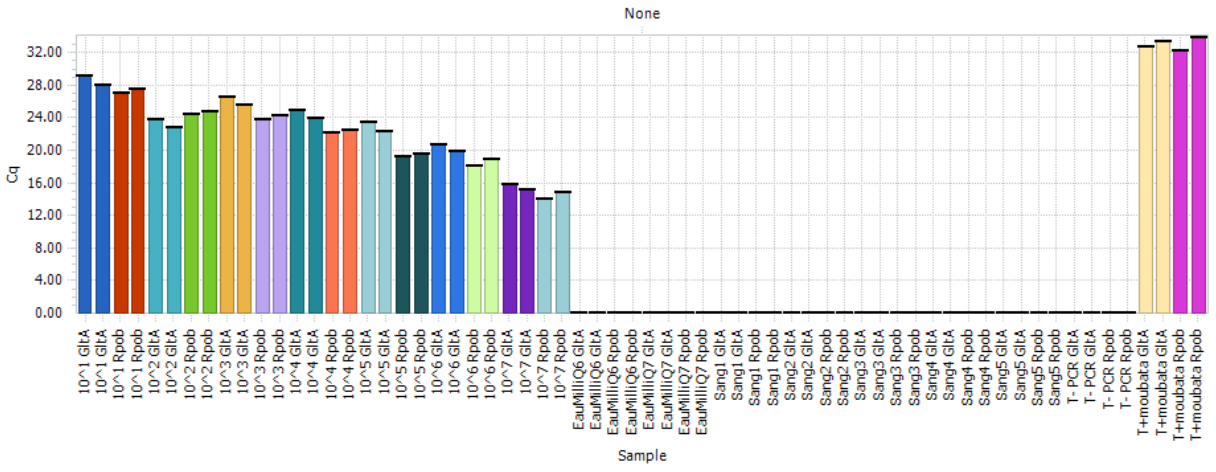
Both male and females were engorged together for reproduction but only females were monitored then (blood meal being facultative for males, we could not be sure if they took antibiotics or not). This was clarified in the text. (Line 156-165)

L103-104: Can the process of blood feeding be a source of potential DNA or microbial contamination that would impact the study? Were there adequate precautions taken?

This comment concerning a potential microbial contamination during the blood feeding is relevant. Blood feeding could be a source of contamination in case of septicaemia from the cow. It is useful to mention here that the blood is sampled from a unique cow which is present on the site of our laboratory ("Ethics" subpart in material and methods concerns this cow). As a consequence, all treatments or health issues concerning the cow were known. No such issue happened during the time of the experiment.

Even if this kind of contamination would not have significant impact on our study and scientific objectives, we tested this potential microbial contamination from the blood samples used for the study. A sample of 1mL of blood had been collected and frozen at -80°C for analyses for each blood meal performed. Those blood samples have been extracted and qPCR have been performed to look for bacterial contamination. A sentence was added in material and methods to mention and detail this control. (Line 201-204)

Please find here the results of the qPCR performed on the blood, as well as the qPCR performed on milli-Q water.



This graph presents the threshold values obtained after qPCR targeting Rickettsia (GltA) and FLE (RpoB) in blood and Milli-Q water samples used in the experiments performed. For each sample, there is two values of Cq, first one for the tick actin gene, second one for the symbiont gene (GltA or RpoB). Standard curve points from the dilutions of a plasmid (samples 10^1 to 10^7) and positive control (T+moubata) were positive. Blood samples (Sang1 to Sang5), Milli-Q water samples (EauMilliQ6 and EauMilliQ7) and negative control (T-PCR) were negative.

L110-112: Are the antibiotics known to have an effect on the 2 targeted endosymbionts? Please specify somewhere in the text.
Yes they are and this parameter was taken into account for the choice of the molecules. This part was moved from discussion to material and methods. (Lines 136 – 142)

L113, L147: Was the Milli-Q water UV treated or treated in order to avoid DNA contamination? Would not molecular grade water be more adequate to further use a DNA-based molecular approach?
Milli-Q water used as a control added in the blood meal instead of vitamins or antibiotics was not UV treated. Extraction + qPCR and qPCR alone were performed on samples of Milli-Q water to rule out contaminations by FLE or *Rickettsia* (see above).
Water used during extractions and qPCR was nuclease-free water with extraction and qPCR negative controls performed (nuclease free water instead of DNA). A mistake was corrected in the preprint where Milli-Q water was mentioned instead of nuclease-free water. (Line 201)

L156: It would be more informative to give a range of DNA concentration than the volume.
The reviewer remark is relevant. However, while our study focused on both endosymbionts FLE and Rickettsia, we did not measure DNA concentrations as they were not representative of the symbiont DNA concentration (which is very small in regard of tick DNA).

L358: There is a typing mistake: "target*ed".
This has been fixed in the revised version of the manuscript (line 136)

L358-361: This information should be put earlier in the text to understand the antibiotic choice.
This has been moved to the material and methods as suggested in the comment above.

Figures 2, 3, 4: This is a minor comment but instead of writing "boxplot of", "histogram of", the authors may directly write the title, for instance: "Ratios of DNA concentration…".
This query has been addressed in the new version of the manuscript.

Reviewer#1: Tuomas Aivelo

Taraveau et al are presenting a preprint on the study that they did on a soft tick species. The authors fed both adult and nymphs with a treatment of two different antibiotics and supplemental vitamin B and measured both the effects on the survival and the fitness of the adult ticks, but also the dynamics of two endosymbiont species within nymphs. They found that antibiotic treatments reduced the endosymbionts but also increased tick survival, whereas vitamin B supplements did not have a drastic effect.

The general problem with the tick microbiome studies is that the effect of the microbiome structure on the tick fitness is difficult to assess and would require laboratory studies such as this one to really tease apart the effects of different community members of tick microbiota. This study follows the traditional path of so-called population perturbation studies, where ecologist have administered antibiotics or antihelminths to different animals and seen how the community changes and what effects this has on the host fitness and survival. Thus, I see this study as valuable in providing important basic knowledge about specific members of the tick microbiota.

I found the study setting and analysis methods sound and reliable – and especially they are very thorough in covering different important aspects, such as the actual changes in endosymbiont abundance. I had a look at the raw data and the analysis scripts and they seemed ok. The one major concern that I have, and which maybe is not even major, but rather medium-sized, is about the structure of the discussion.

Currently, the reading of discussion is a bit arduous task. The discussion has a long passages (rows 338-372, 386-387) that are better suited to other parts, such as introduction or methods. It does not go to the heart of the studied issue, but rather meanders its way. The authors describe it as ”interesting result” that endosymbionts increased in concentration after tick blood meal, though intuitively it seems like a sensible thing for endosymbiont to do. Similarly, the authors say that the antibiotic that the Rickettsia endosymbiont is resistant to did not affect the bacteria, whereas the antibiotic that the both endosymbionts are susceptible to did decrease endosymbiont DNA concentration. Again, hardly interesting in the grand scheme of things. The truly interesting stuff starts at row 419, where the authors go to the discussion about the things related to the title of the manuscript. I agree with the authors discussion on their results here and there are some worthy observations. Indeed, the ticks have long lifecycles and they can also be influenced by the egg composition, for example. I would appreciate if the authors also outline here how they think that a better study which would take into account the whole lifecycle of a tick could be carried out.

Thus, my main feedback would be restructuring the discussion to highlight the core of the manuscript. The main findings can also be brought more to the forefront. There are also worthy technical and methodological comments, but they are now rather scattered. It would make sense to collect these together under a heading such as ”Implications to future studies” or so after the main discussion.

We would like to thank the reviewer for his positive answer and comments. We reorganised the discussion to highlight the core of the study. As rightly suggested, a new part “Implications to future studies” was added in the new version of the manuscript. Comments about the experimental design and propositions for the future were moved into this part. We also shortened or moved in the right place the paragraphs that were making the discussion longer than necessary.
Discussion about the increase in the symbiont load after bloodmeal was kept as it confirms previous results published in the literature, but shortened as it is not the main point of our study.

Minor issues:

- **The title: I found the title a bit too long. Obviously scientific articles are always aiming for new insights, so it is a bit redundant. Maybe just ”Influence of endosymbionts on the reproductive fitness of the tick Ornithodoros moubata” (I am not a native speaker of English so maybe run that also through somebody who has a certain grasp of articles.)**

We agree with the reviewer comment and we modified the title accordingly.

- **There is first a division of bacteria to environmental, primary endosymbionts and secondary endosymbionts, but other bacteria than primary endosymbionts are not much described. I think that both for the symmetry of the description and as a justficiation of your approach, you should also outline what environmental bacteria and what secondary endosymbionts are and especially why you are choosing primary endosymbionts as your target.**

The reviewer remark is relevant. Details have been added in the introduction part (Line 46-56) to explain why we focus our study on primary endosymbionts.

- **Materials and methods: You have a different order of adults and nymphs here than in results. To make the article more readable, consider the order and consistency carefully. Similarly, in results, the end result (i.e., females in the end of reproduction) are shown first and then the reproduction metrics. I understand this order from the point of the clarity, as the endosymbiont DNA concentrations lay ground to your discussion later but that would need a restructuring of the methods.**

We agree with the reviewer comment. We thus changed the text in the materials and methods part to match the order 1) DNA concentration in both nymphs and adult females 2) reproduction metrics in females. A small part was added at the beginning to give an overview of the experimental design. Information about blood meals and treatments were kept on top as they concern all experiments.

- **Rows 101-: I found the description of your protocol a little bit difficult to follow, whereas the figure 1 was clear. I suggest referring to Figure 1 earlier. Similarly, it is not clear how nymphs are selected: it seems that those are from the same adults as previously treated, but how do you make sure that the previous treatment does not affect these results?**

The nymphs are not offspring of the adults used here. They are a completely independent group of individuals for which the parents never received any treatment. We reorganized the section materials and methods to clarify this point. Figure 1 was also mentioned earlier as suggested by the reviewer.

- **Row 181: I would expect that qPCR results would be quite comparable across persons doing the analysis. Was the effect size of this effect a substantial?**

As pointed out by the reviewer, we were also expecting the results to be quite comparable across users. However, the effect associated with the user was consequent and needed to be included in the analyses (GltA: p=1.602×10e-05, F-value=19.17 and, RpoB: p=1.144e×10-65, F-value=472.7).
The fact that the experiment was separated in time was due to laboratory supply shortage during the COVID pandemic which led us to start the analyses on only a few conditions while keeping the rest of the ticks at -80°C.

- **Row 184: A standard formulation would be 1.602 x 10-5 and so on.**

This query has been addressed in both “Materials and methods” and “Results” parts.

- **Rows 184, 212, 213, 227, 228, Tables etc.: Consistent and sensible rounding of decimals is needed.**

This was homogenised with three significant figures.

- **Rows 208-216: I find it unnecessary to present here unstandardized results at all.**

The goal of presenting this result was only to indicate the bloom of symbionts after blood meal. We suggest to keep this part.

- **Tables and figures: The use of statistical difference codes in tables is unnecessary as readers probably understand the numbers faster than figuring out what these asterisks mean. Thus, I would think that the gray background is enough to indicate a single level of significance. Then again, in figures, I would appreciate indications of statistical significance.**

Asterisks have been deleted from the tables. Contrary to the reviewer suggestion, we prefer to keep statistical significance into the tables rather than adding to the figures as some figures would become difficult to read with this statistical indication.

- **In results: I would consider the method of presenting means of different measurements. They are now already in figures as graphically represented and you have also tables on the statistical analysis. For example, the first 50 days, when there is only a one significant variable, you could survive with a lot less of body text.**

We agree with the reviewer comment, body text has been shortened in the result section when the data was already presented in the form of figures, especially for the first 50 days of monitoring.

- **Row 436: I am not fan of describing non-significant results as almost significant. If you decide on an analysis which introduces a clear cut-off, then you have to live with clear cut-off. So I would not "consider carefully" those, but just not consider at all.**

We agree with the reviewer comment and this part has been removed in the new version of the manuscript.

- **Table S1 would make sense to be included in the main manuscript. It could also be incorporate to Figure 1.**

As our preprint already presents a lot of data in the form of tables, we prefer to keep this table in the supplementary materials. The Figure 1 takes into account the number of ticks used in each experiment.

Reviewer#2: Luciana Raggi Hoyos

A series of experiments over the endosymbiont community are performed, trying to evaluate endosymbiont absence after antibiotic treatments and the addition of B vitamins. I would not call "microbiota" these two types of endosymbionts.
The main problem of the study is that they could not get rid of the total endosymbionts and that the experiment was in a short time (50 days). However, observations might be useful for future experiments.
Perhaps information goes better for a short communication.
We would like to thank the reviewer for her positive and relevant comments. There is no specific short communication format in PCI but in response to all comments, we thus shortened both the introduction, results and discussion.

Punctual suggestions:

27-28 - for ticks --> during the tick reproduction cycle
This has been fixed in the new version of the manuscript. (Line 27)

55 – Alimentation
This was replaced by nutrition. (Line 61)

62 - (Guizzo et al. 2017; Li, Zhang, et Zhu 2018; Zhang et al. 2017;
Zhong, Jasinskas, et Barbour 2007; Kurlovs et al. 2014) --> Maybe to cite reviews?, list of references is a bit long.
We agree with the reviewer comment. However, we consider that these references are essential for our article as they deal with the same topic, yet on other species with other methods. We thus chose to quote all of them together before using them at different points in the article.

65 - Repeated line - "poorly known" as in line 45 "microbiota species remains unclear"
This has been removed in the new version of the manuscript.

66 - short blood feedings ?? Is it correct ¨short¨?? *
In soft ticks, the blood meal is short (3h) compared to hard tick species (several days). This part of the sentence has been replaced by "brief blood meal".

67 - 69 - re-write sentence
This sentence has been modified in the revised version of the manuscript. (Line 70-72)

69-71 - make one sentence from those 2
This is done. (Line 73-74)

69-73 - these could be the first lines of the introduction
We fully understand the reviewer suggestion. However, it was decided to focus the introduction on the microbiota instead of the vector which is a model for studying the microbiota. Information on the vector are essential but are not the main focus of this preprint. We consequently prefer to keep this order.

81-83 - creo queue están de más
We agree with the reviewer remark and we shortened this part of the introduction. (Line 84)

89 - such a disruption --> such disruption

This query has been addressed in the revised version of the manuscript. (Line 92)

89 - vitamin B
This is done. (Line 92)

90 - to identify whether part of the...or it t was a consequence of their nutritional role
This is done. (Line 93)

98 - since --> until
Ticks received blood meals before they moulted into adults but did not receive anymore blood meal since they moulted into adults. The sentence has been modified to clarify this point. (Line 100)

102 - How are ticks engorged?
Ticks were engorged from cow blood with an artificial feeding system. 9mL of blood were put into a well from 6-well cell culture plates. Ticks access the blood after biting through a paraffin membrane placed upon the well containing the blood. The sentence has been modified to clarify this point. (Line 124-125)

107 - How many treatments did you have?
Two consecutive treatments were performed in adults. This was clarified in the Material and Methods part. (Line 108 and 156)

108 - following a modified protocol (Duron et al. 2018) where the dilutions...
This is done (line 129)

115 group --> groups
This query has been addressed in the new version of the manuscript. (Line 160)

120-123 - These lines are maybe results
This information was collected to assess if the treatment would affect the success of blood feeding. If so, it would have been more difficult to interpret variations on the reproductive parameters as they could have been consequences of a poor success of blood feeding in treated group instead of consequences of the elimination of the symbionts.
This paragraph has been improved to better highlight its importance as a control. (Line 166-168)

159 - B --> ß
This is done. (Line 185)

197 - B vitamin --> vitamin B or B vitamins
This is done. (Line 236)

354-357 - if it was ruled out I think more information is not needed --> Although, broadly used before,...
This paragraph has been removed.

358 - target --> targeted
This is done. (Line 136)

366 - for ticks or for this species?
This concerns this specific species (Vitamins which are provided by *Francisella*-like endosymbionts in *Ornithodoros moubata*), "for *Ornithodoros moubata*" has been added to clarify this point. (Line 144)

368-372 - It is repeated in the introduction, take it off here or there, but as there were not really changes with vitamin I do not see the point to put it in here.
This has been removed from the discussion.

374 - I think you should re-state the results.
The discussion has been rearranged, results reappear earlier in the text.

381 - those --> these
This is done. (Line 396)

392-299 - these are results, not discussion
We agree with the reviewer comment and we removed this paragraph in the discussion part.

403 - symbiont --> symbionts
This is done. (Line 379)

406 - to eliminate completely the microbiota --> Are you talking about general microbiota that you did not measure? or about these endosymbiotic community that you are analyzing?
This refers to the endosymbiotic community studied here (FLE and Rickettsia). The sentence has been modified. (Line 382)

412-418 - It is not clear, re-write
We modified this paragraph.

423 - I see in Fig 3 symbionts were indeed eliminated with
This sentence refers to the first treatment out of two successive treatments. After this first treatment, we are not completely sure that the symbionts were fully eliminated as we could not analyse the ticks (still needed for the second treatment)

432-433 - bacterias --> bacteria
This is done. (Line 408)

434 - Rickettsia is abundant but after Perez-Sardiña et al (2023) FLE do not seem to be very abundant
The study published by Perez-Sardiña *et al* focuses on the microbiota present in both salivary glands and midgut. FLE are mostly predominant in ovaries and malpighian tubules (Duron *et al*. 2018). It is also important to note that the microbiota can change from one tick colony to another, especially after long times in the laboratory. Ticks from the CIRAD colony were analysed by sequencing in previous work which revealed the high prevalence of FLE and *Rickettsia*.

438-440 - needs reference
After rearranging the discussion, this part has been removed.

473 - I think this line should be in the introduction and in methods to clarify this since the beginning.
This was added in Material and Methods. (Line 108)

Other observations:
1. I would shorten the introduction. Some information could be more useful in discussion as the 57-58 lines. There is more information than needed.

Thank you for your comment, several parts of the introduction have been shortened, some information were moved to the discussion section.

2. I think you should point out somewhere in introduction that Francisella synthesize B vitamins that are deficient in the blood meal of ticks and that it is maternally transmitted to all maturing tick oocytes.
This was added in the new version of the manuscript. (Line 81)

3. You state that this work is focused on the microbiota --> you are not really studying microbiota, but endosymbiont community. There is a paper on O. moubata microbiota (Perez-Sardiña et al 2023, doi: 10.3389/fmicb.2023.1173609), it would be interesting to analyze that data.
Indeed, we focus on the two main endosymbiont communities which are part of the microbiota of our strain of *Ornithodoros moubata*. Yet, the treatment performed here probably impacts the whole microbiota of the tick, which must be considered during analyses. We tried to review the use of the term microbiota in the manuscript to replace it by FLE and Rickettsia when appropriate. The title of the manuscript has also been modified. As suggested by the reviewer, we also included the reference to Perez-Sardiña *et al*.

4. Experimental setup is not clear enough, I would state clearly which is Experiment 1 and 2 and treatments in each experiment.
We rearranged the material and methods section for more clarity.

5. Cites have a wrong (e.g. Duron et Gottlieb 2020) format with "et" word --> &
This has been fixed in the new version of the manuscript.

6. It is necessary to review English grammar.
Grammar review was performed on the new version of the manuscript.

7. One interesting conclusion is that endosymbionts are not easily lost, and the it is important to analyze de whole microbiota.
We agree with the reviewer comment and this was added in the discussion. (Line 471-475)

8. Were statistically significant differences determined for each treatment by post hoc tests (Kruskal-Wallis and Mann-Witney-Wilcoxon)? As graphs are not labeled I suspect there are no differences between treatments anywhere.
Post-hoc analysis were performed using the function emmeans with the Tukey HSD test to identify which groups were statistically different from one another. This was added in the text for group comparisons and in the material and methods section (Line 233-234).

Fig 3: it is not clear at which point of the experiment these data were taken
This has been clarified in the legend of the figure and in the material and methods part. (Ticks tested in Figure 3 are the female adults which were still alive after the two consecutive antibiotic treatments and two 50 day monitoring of the reproduction)

Fig 6: Is this graph including nymphs and adults or is only adults? what happens if you separate? do you see the same pattern in adults separated from nymphs?

This graph only includes the adult females. Nymphs were killed for qPCR analyses and could not be used to analyse the survival rate. The legend of the figure has been improved to clarify this point.